FEBS 16949
FEBS Letters 385 (1996) 11–14

A molecular map of titin/connectin elasticity reveals two different mechanisms acting in series

Mathias Gautel*, David Goulding

European Molecular Biology Laboratory, Biological Structures Division, Postfach 102209, 69012 Heidelberg, Germany

Received 11 March 1996

Abstract **In the I-band of skeletal muscle sarcomeres, the elastic region of titin consists of immunoglobulin (Ig) domains, and non-modular regions rich in proline, hydrophobic, and charged residues (PEVK). Using immunoelectron microscopy with sequence-assigned monoclonal antibodies, we demonstrate that extension of the Ig regions in M. psoas occurs largely at sarcomere lengths between 2 and 2.8 μm, decreasing in slope towards higher lengths. The Ig domains do not unfold. Above 2.6 μm, length changes are increasingly due to the PEVK-rich regions. We therefore propose that rubber-like properties of the PEVK-rich regions are mainly contributing to skeletal titin elasticity.**

Key words: Titin; Connectin; Muscle elasticity; Ultrastructure; Immunoelectron microscopy; Monoclonal antibody

1. Introduction

Titin [1], first described as connectin [2,3], is the giant sarcomeric protein of striated muscle. In the I-band, it forms an elastic connection between Z-discs and thick filaments [2-7]. Its elasticity is considered the basis of the passive tension of muscle [1-3]. The elastic region of skeletal titin is composed of contiguous stretches of immunoglobulin domains, and non-modular regions consisting mainly of proline, hydrophobic, and charged residues (PEVK-rich) [8]. An important question is what the contributions of the diverse secondary structure elements to the elastic response are. Previous investigations in skeletal and cardiac muscle indicated that different I-band segments extended differentially [5-7,9]. However, no correlation could be made between the sequence elements of I-band titin and its extension, because the molecular position of the antibodies used in these studies was unknown. Unfolding of Ig domains was proposed as the main mechanism of tension generation [10], which would create great length increases in the tandem Ig regions.

To investigate the contributions of the various molecular regions of skeletal I-band titin to sarcomere extension within physiological limits, and to gain information on the size of individual Ig domains under stretch, we used an ultrastructural approach by immunoelectron microscopy with new, sequence-assigned monoclonal antibodies.

2. Materials and methods

2.1. Protein expression and antibodies

Titin domains from the regions of interest (MG1: EMBL X90569, bp 15671–16516; MG2: bp 77–339) were expressed in *E. coli* and purified as described [11]. BALB/c mice were immunised in a standardised scheme and spleens from reactive animals fused with NSO/1 myeloma cells as described [12]. Hybridoma lines were selected and subcloned as described [12]. Specificity was assayed in Western blots of total rabbit M. psoas extracts as described [4] and in blot assays using a panel of recombinant titin I-band domains [13] to assay for intramolecular cross-reactivity. The antibodies MG1 and MG2 belong to the IgG1 subclass and were found to be specific for titin, to recognise a single defined epitope on recombinant titin domains and to be paraformaldehyde-resistant. T12 [4] was a generous gift by D. Fürst.

2.2. Immunoelectron microscopy

Immunoelectron microscopy was performed essentially as described [14]. Psoas muscle was excised from freshly killed rabbits on ice, single fibres dissected, stretched in relaxing solution [14,15] and fixed with 4% paraformaldehyde. Processing for cryosectioning and immunolabelling with 10 nm gold particles was as described [14]. The centre of the immune complex identified by the gold particles was used as the measuring point. The tandem Ig region was determined as the distance between the Z-disk centre and the antibody label; the PEVK-rich region was determined as the distance between the antibody label and the thick filament. The thick filament length of 1.57 μm [16] was assumed as reference length to determine the PEVK-rich region when the last crossbridge was not identifiable.

3. Results

3.1. Molecular mapping of the elastic regions of titin

The movement of titin domains at the transitions between the contiguous immunoglobulin domains (tandem Ig) and the PEVK-rich regions [8] (Fig. 1) was investigated by immunoelectron microscopy in rabbit psoas muscle. We used two monoclonal antibodies, the first (MG1) raised against the constitutively expressed junction between the differentially spliced tandem Ig and PEVK-rich regions [8] (Fig. 1). The second (MG2) specifically binds the boundary between the constitutively and differentially expressed tandem Ig regions [8]. The extension of the tandem Ig regions was measured from the distance of the MG1 epitope relative to the Z-disk. The extension of the PEVK-rich region together with a short stretch of 22 tandem Ig domains was measured as the distance from MG1 to the end of the thick filament. Immunolocalisation of the monoclonal antibodies T12 [4] and MG2 was used to make defined measurements in the tandem Ig region. T12 binds at the beginning of the constitutively expressed tandem Ig region [8] near the Z-disk (Fig. 1) and is preceded by non-elastic sequences [4,9]. The distance between T12 and MG2 is 13 Ig domains.

We found that there is a differential movement of the MG1 epitope dependent on sarcomere length in rabbit psoas muscle

*Corresponding author. Fax: (49) (6221) 387 306;
E-mail: Gautel@EMBL-Heidelberg.DE

PII S0014-5793(96)00338-9

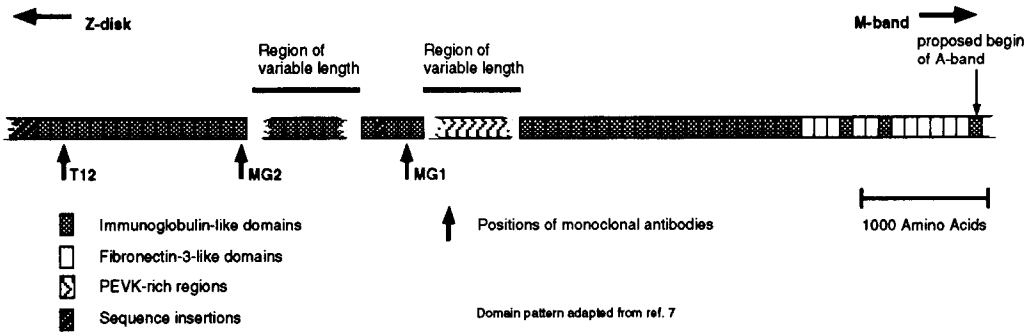
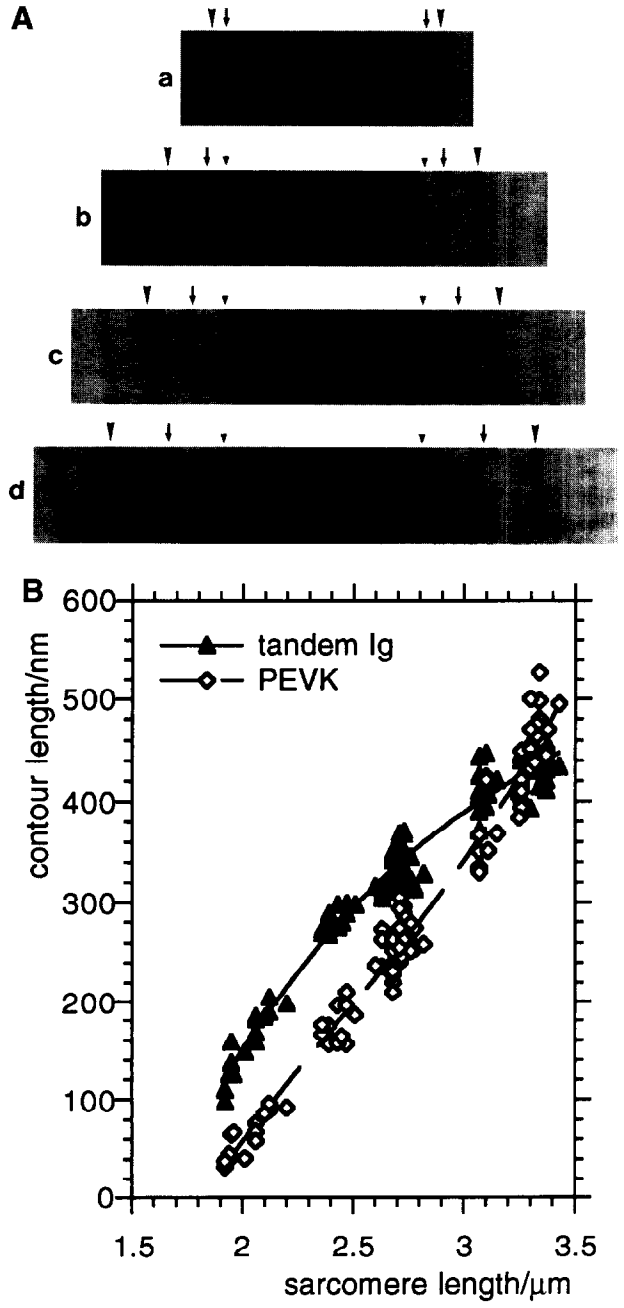


Fig. 1. Positions of the epitopes of the antibodies for this study on the domain pattern of skeletal I-band titin (adapted from [8]). The regions of variable lengths due to differential splicing are marked.



(Fig. 2a,b). At small sarcomere lengths up to 2.8 μm, the movement of the MG1 epitope to the Z-disk dominates sarcomere extension. The passive tension is low in this region [14,15]. The extension of the tandem Ig region was approximately linear along sarcomeric length ranges of up to 2.6–2.8 μm, where resting tension is low [14,15]. The tandem Ig regions can therefore be extended to a linear arrangement with a relatively low energy cost. This signifies that the interactions between domains of the poly-immunoglobulin chains of titin are very weak, a finding also observed in vitro with recombinant fragments from this region [13]. At 3 μm, the slope of the extension of the immunoglobulin chain decreases strongly (Fig. 2b). At this sarcomere length, the approximate dimensions of single immunoglobulin domains under physiological strain can be calculated. The distance measured between the constant epitopes of the monoclonal antibodies T12 and MG2 is 43 ± 9 nm (Fig. 2c). Each of the 13 Ig domains between both epitopes is therefore about 3.3 nm long.

At sarcomere lengths beyond 2.8 μm, the movement of the

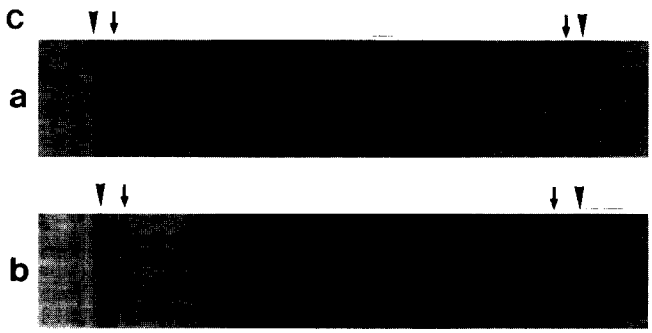


Fig. 2. Differential movement of the MG1 epitope during the extension of rabbit M. psoas. (a) Immunoelectron microscopical visualisation by nanogold beads of the bound titin antibody MG1 at various sarcomere lengths. At 1.82 μm, the label is sharply localised near the A-band end. a, 1.82 μm; b, 2.44 μm; c, 2.79 μm; d, 3.33 μm sarcomere lengths. Bold arrowheads: Z-discs. Light arrows: thick filament ends. Arrows: position of immunolabel. Magnification 25 800×. (b) The position of the immunolabel in (a) was determined, and plotted against sarcomere lengths (n = 110). The extension of the tandem Ig region precedes that of the PEVK-rich regions, and approaches a plateau at higher sarcomere lengths. (c) Position of T12 (a) and MG1 (b) in stretched sarcomeres. The positions of both antibody labels are separated by approximately 43 nm. Bold arrowheads: Z-discs. Arrows: position of immunolabel. Magnification 26 114×.

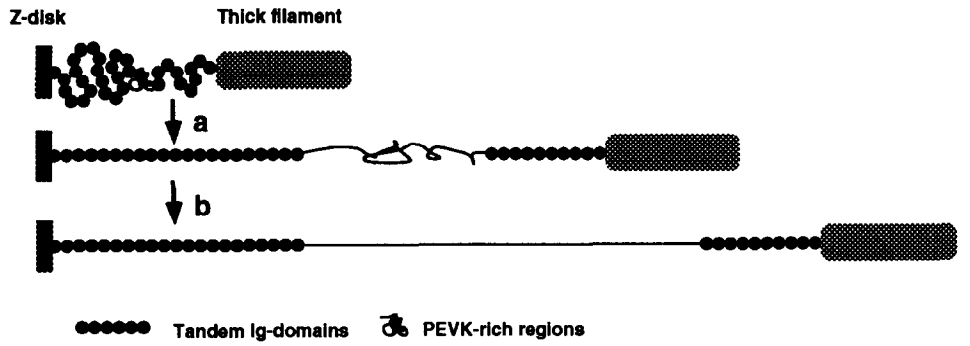


Fig. 3. A model for the sequential extension of titin I-band structures. (a) At small sarcomere lengths, the initially coiled up 'string of beads' of tandem Ig domains is straightened. Some extension in the PEVK-rich region may take place. (b) At higher sarcomere lengths, where muscle generates passive tension, the PEVK-rich region becomes increasingly extended whereas the extension of the tandem Ig region levels off. The slope of the passive tension curve would be expected to approach infinity when the tandem Ig regions are fully stretched; further extension is then only possible by extension of the PEVK-rich regions and finally by destruction of anchorage [14].

MG1 epitope compared to the end of the thick filaments dominates sarcomeric extension (Fig. 2a,b). Above 2.6 μm, there is an exponential increase in resting tension in M. psoas [14,15]. Whereas the extension of the tandem Ig region levels off at higher sarcomere lengths (Fig. 2b), the PEVK-rich segment is extended further linearly (Fig. 2b).

4. Discussion

Using ultrastructural investigations of rabbit psoas muscle, we have mapped the extension of the titin molecule and resolved its two major molecular components in the I-band, the tandem Ig region and the PEVK-rich regions [8] (Figs. 1 and 2). We observed that the tandem Ig region mostly contributes to length changes at smaller sarcomere lengths and therefore under low resting tension. Using a pair of monoclonal antibodies (T12 [4] and MG2) in the constitutively expressed tandem Ig region, we have shown that the dimensions of single Ig domains in stretched sarcomeres do not significantly exceed 4 nm even at high sarcomere lengths (Fig. 2c). This figure is very close to the dimensions of a folded titin domain in solution structures [17]. Therefore, it is unlikely that the titin Ig domains unfold as sarcomere length increases as previously proposed [10]. On the contrary, the stability even for isolated single titin domains has been observed to be generally very high [13,18]. Also, the physiological working range in rodents does not exceed actomyosin overlap in situ and does not extend beyond the sarcomere lengths we investigated (Goulding et al., unpublished observation). The tandem Ig regions are therefore expected to counteract external force only once the domain chain is fully extended. This point is reached at about 3 μm sarcomere length in M. psoas and is near the upper limit of the physiological working range of the sarcomere [14,15]. Above this sarcomere length, there is no further significant extension of the tandem Ig region (Fig. 2b). The extension of the PEVK-rich region C-terminal to the MG1 epitope is therefore linked to exponential passive tension generation in skeletal muscle, identifying this region as the main generator of passive tension.

The proline-rich PEVK sequence is predicted to adopt a random conformation (A. Pastore, personal communication), its hydrophobic residues being shielded from solvent in a 'collapsed' state. A collapsed state is supported by the very short distance between the MG1 epitope and the thick filament at short sarcomere lengths (Fig. 2a,b). Extension of such a collapsed state can firstly account for significant length increases [19] by linear extension of the polypeptide chain. Secondly, the resulting exposure of hydrophobic residues to cytosolic water is an energy-consuming step [20,21]. Exposure of hydrophobic residues would create a significant entropic driving force [20,21] of the 'unfolded', extended form of the PEVK-rich segment to provide a strong spring that would recoil when external strain is released. Indeed, previous biomechanical studies have shown that passive tension of skeletal muscle shows a temperature-dependent behaviour expected for an entropic mechanism involving the exposure of hydrophobic side chains [15]. Cooperative charge interactions between the glutamic acid and lysine residues may provide further attractive forces during 'refolding' of the PEVK-rich segment [22]. Our findings describe the differential extensibility of skeletal titin previously observed [5-7] on a molecular basis and link distinct mechanical functions to the as yet uncharacterised primary structure elements of skeletal I-band titin. At short sarcomere lengths, the Ig domains are responsible for increases in length between the Z-disc and the thick filament (Fig. 3a). At longer sarcomere lengths, the PEVK elements take over, and enable further extension of the sarcomere (Fig. 3b). This simplified model may apply only to the less complex skeletal titin, consisting of only one isoform [8]. Furthermore, extension is also observed in the PEVK-rich sequence at low sarcomere lengths, and in the tandem Ig region at higher sarcomere lengths. Although both mechanisms act in series, they are not entirely separate. Finally, different lengths of differentially spliced tandem Ig stretches could modulate the sarcomere lengths at which the tension-generating PEVK elements are activated in different muscles.

Acknowledgements: We are greatly indebted to Fricke Pietruschka for monoclonal antibodies. We are further most grateful to Belinda Bullard, Annalisa Pastore and Kevin Leonard for continuous support of this work and stimulating discussions, to Pauline Bennett and Michelle Peckham for discussion of EM results and critical reading of the manuscript, and to Siegfried Labeit for communication of the titin sequence prior to publication and continuous stimulation.

References

[1] Wang, K., McClure, J. and Tu, A. (1979) Proc. Natl. Acad. Sci. USA 76, 3698–3702.

[2] Maruyama, K., Natori, R. and Nonomura, Y. (1976) Nature 262, 58–59.
[3] Maruyama, K., Matsubara, S., Natori, R., Nonomura, Y., Kimura, S., Ohashi, K., Murakami, F., Handa, S. and Eguchi, G. (1977) J. Biochem. 82, 317–337.
[4] Fürst, D.O., Osborn, M., Nave, R. and Weber, K. (1988) J. Cell. Biol. 106, 1563–1572.
[5] Itoh, Y., Suzuki, T., Kimura, S., Ohashi, K., Higushi, H., Sawada, H., Shimizu, T., Shibata, M. and Maruyama, K.J. (1988) Biochemistry 104, 504–508.
[6] Funatsu, T., Kono, E., Higuchi, H., Kimura, S. Ishiwata, S., Yoshioka, T., Maruyama, K. and Tsukita, S. (1993) J. Cell Biol. 120, 711–724.
[7] Horowits, R. and Podolsky, R.J. (1987) J. Cell Biol. 105, 2217-2223.
[8] Labeit, S. and Kolmerer, B. (1995) Science 270, 293–296.
[9] Trombitás, K. and Pollack, G.H. (1993) J. Muscle Res. Cell Motil. 14, 416- 422.
[10] Erickson, H. (1994) Proc. Natl. Acad. Sci. USA 91, 10114–10118.
[11] Politou, A., Gautel, M., Joseph, C. and Pastore, A. (1994) FEBS Lett. 352, 27–31.
[12] Harlow, E. and Lane, D. (1988) pp. 174–238. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[13] Politou, A., Gautel, M., Improta, S., Vangelista, L. and Pastore, A. (1996) J. Mol. Biol. 255, 604–616.
[14] Wang, K., McCarter, R., Wright, J., Beverly, J. and Ramirez-Mitchell, R. (1991) Proc. Natl. Acad. Sci. USA 88, 7101–7105.
[15] Ranatunga, K.W. (1994) Biophys. J. 66, 1531–1541.
[16] Craig, R. and Offer, G. (1976) J. Mol. Biol. 102, 325–332.
[17] Pfuhl, M. and Pastore, A. (1995) Structure 3, 391–401.
[18] Politou, A., Thomas, D. and Pastore, A. (1995) Biophys. J. 69, 2601–2610.
[19] Pauling, L., Crey, R.B. and Branson, H.R. (1951) Proc. Natl. Acad. Sci. USA 37, 205–211.
[20] Makhatadze, G.I. and Privalov, P.L. (1993) J. Mol. Biol. 232, 639–659.
[21] Privalov, P.L. and Makhatadze, G.I. (1993) J. Mol. Biol. 232, 660–679.
[22] Horovitz, A. and Fersht, A.R. (1993) J. Mol. Biol. 224, 733–740.